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<b>(21) International Application Number:</b> PCT/NL96/00230 <b>(22) International Filing Date:</b> 7 June 1996 (07.06.96)  <b>(30) Priority Data:</b> 08/480,020                      7 June 1995 (07.06.95)                      US  <b>(71) Applicant:</b> AESCULAAP B.V. [NL/NL]; Mijlstraat 35, NL-5281 LJ Boxtel (NL).  <b>(72) Inventors:</b> NOTEBORN, Matheus, Hubertus, Maria; Sternstraat 15, NL-2352 EH Leiderdorp (NL). KOCH, Guus; Wildbaan 12, NL-8222 AG Lelystad (NL).  <b>(74) Agent:</b> SMULDERS, Th., A., H., J.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> NEUTRALIZING CONFORMATIONAL EPITOPES OF CHICKEN ANEMIA VIRUS  <b>(57) Abstract</b> <p>The production of the conformational neutralizing epitope of chicken anemia virus is described and compositions for preventing or treating CAV infections, in particular vaccines less pathogenic than CAV itself. All these compositions were shown to synthesize the conformational neutralizing epitope, necessary for the protective immune response against CAV infections. The invention provides recombinant DNA molecules derived from the CAV genome, and recombinant DNA fragments of the CAV genome integrated in the genome of other virus vectors. In particular, these compositions comprise a sub-unit vaccine, a recombinant live virus vaccine and an attenuated vaccine against CAV infections. The invention also provides the production of neutralizing antibodies directed against CAV and diagnostic kits for the detection of CAV.</p>		

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Title: Neutralizing conformational epitopes of Chicken anemia virus .

#### BRIEF DESCRIPTION OF THE INVENTION

The present invention provides the formation and characterization of the neutralizing conformational epitope of the CAV protein VP1, which is required for eliciting a protective immune response in vaccinated animals.

In particular, the production of neutralizing antibodies against CAV is described. In a diagnostic test, based on these produced neutralizing antibodies, CAV (particles) are detected.

Various vaccine vectors against CAV infections are disclosed. All these vaccines are less pathogenic than CAV. All these vaccine vector are shown to produce the required neutralizing conformational epitope of CAV.

One vector provides a subunit vaccine, based on a recombinant baculovirus expressing both VP1 and VP2 in the same cell. A second type of vaccine is a live virus vector, comprising a Marek's disease vector stably harboring the coding sequences for VP1 and VP2. The third CAV vaccine comprises genetically attenuated CAV strains with a reduced cytopathogenic effect.

#### BACKGROUND OF THE INVENTION

Chicken anemia virus (CAV) is a small virus of a unique type with a particle diameter of 23 to 25 nm and a genome consisting of a circular single-stranded (minus-strand) DNA (Gelder blom et al., 1989, Noteborn et al., 1991 and Todd et al., 1990). This DNA multiplies in infected cells via a circular double-stranded replicative intermediate, which was recently cloned and fully sequenced. The CAV genome is 2319 nucleotides long (Noteborn and De Boer, 1990). DNA analysis of CAV strains isolated in different continents revealed only minor

differences among the various isolates (Meehan et al., 1992; accession number M81223, Claessens et al., 1991; D10068, Kato et al., 1995; D31965 and Pallister et al., 1994 Soine et al. 1993 and 1994; L14767). Recently, on the basis of the genome structure, CAV has been placed into the novel virus family of Circoviridae. However, CAV is not related to the other members of this virus family consisting of animal single-stranded circular-DNA viruses, such as porcine circovirus and psittacine beak-and-feather-disease virus (Noteborn and Koch, 1995).

The CAV genome contains one evident promoter/enhancer region (Noteborn et al. 1994) which regulates the CAV transcription. The major transcript from the CAV genome is an unspliced polycistronic mRNA of about 2100 nucleotides encoding three proteins of 51.6 kDa (VP1), 24.0 kDa (VP2) and 13.3 kDa (VP3 or apoptin (Noteborn and Koch, 1995, Noteborn et al., 1992 and Phenix et al., 1994). All three predicted CAV proteins are synthesized in CAV-infected cells (Noteborn and Koch, 1995). Immunization with (recombinant) VP1 and VP2 synchronously synthesized in the same cells elicits a protective response and can be used as subunit vaccine against chicken infectious anemia (Koch et al., 1995 and Noteborn and Koch, 1994c).

CAV causes clinical and subclinical disease in chickens, and is recognized as an important avian pathogen worldwide (McIlroy et al., 1992 and McNulty et al., 1991). Day-old chicks are especially susceptible to CAV infections. In these animals lethargy, anorexia and anemia are observed from 10 days after inoculation with CAV. After infection mortality may increase to a maximum of 50%. With increasing age the resistance also increases (Jeurissen et al., 1992). The hematocrit values of chicks that had been infected with CAV at an age of 1-3 days are decreased. CAV infections of 1-21 days old chicks result in a depletion of in particular the thymus cortex. However, in older chickens CAV can subclinically multiply.

CAV infection in older chickens can be determined by the occurrence of seroconversion.

The depletion of the cortical thymocytes is considered to cause immunodeficiency resulting in enhanced concurrent infections and to vaccination failures (Noteborn and Koch, 1995). The depletion of thymocytes and most likely also of erythroblastoid cells occur via CAV-induced apoptosis (Jeurissen et al., 1992a). The CAV-encoded protein apoptin is the main inducer of this phenomenon (Noteborn et al., 1994).

Maternal antibodies have been found to give an important protection against CAV infection. Vielitz et al. (1991) reported that hens exposed to CAV from large amounts of antibodies, which are passively transferred to the offspring per ovum. These antibodies protect against chicken infectious-anaemia-associated disease.

CAV-neutralizing antibodies were detected in yolk of eggs produced by hens that had been inoculated with lysates of cells that had been co-infected with CAV-recombinant baculovirus and produced all three CAV proteins, or mainly VP1 and VP2. Specific clinical signs did not develop in CAV-challenged progeny that hatched from these eggs (Koch et al., 1995).

Vielitz and Landgraf (1988) have developed a vaccine against infectious anaemia, which is based on CAV propagated in chicken embryos. Currently, this is the only commercially available vaccine. The offspring of vaccinated breeders are protected against infectious anaemia. Vaccination is possible because when maternal immunity vanished, the birds are susceptible to virus infection without developing the disease. Obviously, the use of live vaccines based on non-attenuated virus harbors risks. Experimental infection of 3-week-old chickens resulted in a comprehensive decrease of the functions of the immune system (McConnell et al., 1993, and 1993a) in the absence of disease. In line with this finding, McIlroy (1992) have provided evidence that CAV also causes

considerable economic losses in the absence of disease:  
the subclinical disease negatively affected feed  
conversion and average body weight and increased  
medication, both of which result in a considerable  
5 financial burden. So far, non-pathogenic CAV has not been  
isolated.

The recombinant CAV proteins synthesized by means of  
the baculovirus expression system can be used as a subunit  
vaccine. The recombinant CAV proteins VP1 and VP2 have  
10 been proven to protect chicks by maternal immunity (Koch  
et al, 1995). Since the baculovirus vector is an,  
insect-specific virus, known to be non-pathogenic for  
vertebrates, it can be cultured and supplied to chicken  
without undue risks (Vlak and Keus, 1990).

15 In general, live-virus vaccines induce a better  
immune response and are less expensive than sub-unit  
vaccines. Therefore, knowledge about the immunogenicity of  
the various CAV proteins can be used to construct  
attenuated CAV or other recombinant-virus vectors, such as  
20 avian herpes viruses (Nakamura et al., 1992, Morgan et  
al., 1993) or fowlpox virus (Nazerian et al., 1992, Boyle  
and Heine, 1993). These recombinant viruses should express  
the CAV protein VP1 and VP2, in addition to their own  
proteins, and may be applicable as vaccine for both layer  
25 breeders and broiler breeders to protect their offspring  
against infectious anaemia. In addition, such vectors may  
be used to protect maternally immune broilers against  
subclinical disease.

### 30 DETAILED DESCRIPTION OF THE INVENTION.

The present invention relates to production and  
analyses of the neutralizing conformational epitope  
structure of chicken anemia virus (CAV).

Furthermore, the production of neutralizing  
35 monoclonal antibodies directed against CAV is disclosed.  
It is shown that these neutralizing antibodies are

directed against a conformational epitope of CAV protein VP1.

Also a diagnostic test kit, based on these neutralizing antibodies, for the detection of CAV will be described. This invention provides evidence for the mechanism by which the neutralizing antibodies neutralize CAV particles.

For the formation of the neutralizing conformational epitope of VP1, the synthesis of VP2 within the same cell is required. Recombinant baculovirus expressing only VP1 in insect cells only does not react with neutralizing antibodies directed against CAV, but these neutralizing antibodies will react when VP1 and VP2 are synthesized in one cell.

In purified CAV capsids, however, only VP1 is present. This invention discloses that during the synthesis of VP1, and most likely during its complex formation resulting in the CAV capsids, VP2 binds temporarily to VP1. Denaturation of the CAV capsids results in the destruction of the neutralizing epitope, indicating that the neutralizing epitope is an conformational one.

Besides, the invention relates to vaccines and compositions for preventing or treating virus infections in poultry, in particular infections with CAV.

In particular, the invention relates to vaccines that are less pathogenic than the CAV itself but are still capable of producing the neutralizing conformational epitope. Vaccination of chickens with these type of vaccines will lead to the generation of neutralizing antibodies and thus protect the animals and their progeny against CAV infections.

The invention relates to a baculovirus vector which contains separately on its genome the coding sequences for VP1 or VP2. This recombinant baculovirus is able to synthesize VP1 and VP2 in the same cell, resulting in the



formation of the neutralizing conformational epitope of CAV.

The invention also relates to the construction of a Marek's disease virus (MDV) vectors containing CAV  
5 sequences encoding VP1, VP2 proteins. In particular, this recombinant MDV vector synthesizes the recombinant CAV proteins in such a way that the neutralizing conformational epitope of VP1 is properly made.

Furthermore, the invention describes the formation of  
10 various attenuated CAV strains, which reveals a reduced cytopathogenic effect in chicken T cells in comparison to a wild-type-derived CAV strain. The attenuated CAV strains were made by introducing point mutations in a cloned CAV DNA genome, in particular a sequence within the  
15 promoter/enhancer region has been mutated. The attenuated CAV mutant strains are able to produce the neutralizing conformational epitope.

Processes for the prophylaxis or control of CAV infections, in particular in chickens, and processes for  
20 the preparation of recombinant parts of CAV comprising sequences, and processes for the preparation of vaccine are also subjects of the invention.

Thus, reiterating the invention provides a neutralizing antibody or an antibody fragment or  
25 derivative thereof reacting with a conformational epitope of viral protein 1 (VP1) of chicken anaemia virus (CAV) recognized by a monoclonal antibody designated as 132-1, 132-2 or 132-3, as produced by the hybridoma's deposited under no.'s xxxxxx, yyyyyy, zzzzzz at the Insitut Pasteur,  
30 Paris, France. A workable experiment for determining whether an antibody is an antibody according to the invention is to see if it cross-reacts (or competes) with an antibody from the deposited hybridomas. Fragments and derivatives of antibodies are well known in the art and  
35 hardly need further explanation for the person skilled in the art.



The invention also provides a conformational neutralizing epitope of viral protein 1 of chicken anaemia virus recognized by an antibody as disclosed above. The epitope may be part of a larger molecule, it may for instance be bound to a carrier or the epitope may be repeated (at intervals) in a polypeptide chain. Preferably the epitope is part of a larger part of VP1, because such a molecule will have the right conformation more easily.

As described the conformational epitope will only be present on VP1 if it is produced in one cell, preferably from one vector together with VP2. Therefore the invention provides a method for producing a viral protein 1 comprising a conformational epitope as disclosed above, comprising the expression of said viral protein 1 in one cell together with viral protein 2 of said CAV whereby the genetic information encoding VP1 and the genetic information encoding VP2 are separately present on one recombinant vector.

The invention also includes vectors for use in a method just described comprising as two separate coding sequences the genetic information encoding VP1 and the genetic information encoding VP2. Preferably such a vector is based on Marek's disease virus vector so that a vaccine can be produced giving protection against two pathogens.

A very safe and efficient expression system for the viral proteins according to the invention are vectors which are based on a baculo virus vector. As explained before Baculovirus is generally regarded as safe for use in vertebrates since it cannot infect them.

The invention also provides another way of arriving at vaccines or vaccine components which do have the very important neutralizing conformational epitope, but have reduced pathogenicity. (Attenuated viruses for instance.)

This is achieved by providing a method for providing a viral protein 1 comprising a neutralizing conformational epitope according to the invention, comprising expressing at least a functional part of VP1 and VP2 from genes

encoding them, at least one of which genes is under control of a regulatory sequence derived from the CAV sequence upstream of the transcription initiation site which regulatory sequence is modified to reduce its efficiency. In this way recombinant viruses can be produced which do not replicate as fast and therefor are less pathogenic (i.e. attenuated).

Such virus particles are also part of the invention. As disclosed hereinbefore the modification is preferably in the promoter/enhancer region and most preferably the modification is in the 12 base pair insert in the promoter enhancer region.

Recombinant virus particles obtainable by any method according to the invention are also part of the invention, as are nucleic acids for use in any method according to the invention, for instance vectors comprising a gene encoding at least a functional part of VP1 and a gene encoding a functional part of VP2, at least one of the genes being under control of a regulatory sequence which is modified to reduce its efficiency.

The antibodies and epitopes according to the invention are also useful in a diagnostic test kit for detecting or determining the presence of CAV or antibodies to CAV in a sample

Furthermore vaccines for the treatment or prophylaxis of CAV associated disease comprising an antibody or an epitope according to the invention together with a suitable adjuvans and/or a suitable vehicle for administration are also provided herewith as are vaccines for the treatment or prophylaxis of CAV associated disease comprising recombinant virus particles according to the invention together with a suitable adjuvans and/or a suitable vehicle for administration.

The invention will be explained in more detail on the basis of the following experimental part. This is only for the purpose of illustration and should not be interpreted as a limitation of the scope of protection.

## EXPERIMENTAL

Purification of CAV particles to elicit neutralizing monoclonal antibodies against CAV

5 For the production of neutralizing monoclonal antibodies against CAV, mice were injected with purified CAV particles.

The supernatant of a 1-liter culture of CAV-infected MDCC-MSB1 cells was forty times concentrated by means of  
10 a MILLITAN 300-kDa filter (Millipore, USA). The supernatant was dialyzed against 10mM Tris(ph 8.7)-1mM EDTA (TE) buffer. Subsequently, sodium dodecyl sulphate (SDS) to an end-concentration of 0.5% was added to the CAV-capsid suspension and incubated for 30 minutes at  
15 37°C. Finally, the CAV capsids were pelleted on a 30% sucrose cushion. The pellet containing the CAV capsids was resuspended in 1 ml TE buffer. Mice were twice injected with 100 µl CAV-capsid suspension.

20 In-vitro neutralization test.

The supernatants of the candidate monoclonal antibodies were diluted 1:2 and then a two-fold dilution series was made. The diluted supernatants were incubated for 1 hour with 104-105 TCID50 CAV-Cux-1 (Von Bülow et  
25 al., 1983, Von Bülow, 1985). Approximately one hundred thousand cells of the T cell line MDCC-MSB1 transformed by Marek's disease virus were infected with this mixture of diluted supernatant and virus (Yuasa, 1983, Yuasa et al. 1983).

30

Immunofluorescence and immunoperoxidase assay

Cells were fixed with 80% acetone and used for immunofluorescence assays with CAV-specific monoclonal antibodies and goat anti-mouse IgC conjugated with  
35 fluorescein, as described by Neteborn et al. (1990).

Recombinant-Vp1/VP2-MDV-infected CEF were used for immunoperoxidase staining with CAV-specific monoclonal antibodies, as described by Jeurissen et al. (1988).

5 Enzyme-linked immunosorbens assay (ELISA)

Microtiter wells (Greiner, FRG) were coated with the CAV-specific neutralizing monoclonal antibody 132.1, which was 1:10,000 diluted in 50 mM sodiumbicarbonate pH 9.6. Wash the wells three times with tapped water containing  
10 0.05% Tween 80. Saturate the wells with 100 µl phosphate-buffered saline containing 4% horse serum, 51 gram/liter NaCl, and 0.05% Tween 80 (saturation buffer) for 30 minutes at 37°C. Wash the wells three times with tap water containing 0.05% Tween 80. Next, 50 µl of  
15 non-diluted chicken serum and 50 µl of thirty times concentrated supernatant containing CAV particles, or 50 µl of a lysate of insect cells containing recombinant VP1 and VP2 proteins, were mixed, added per well and incubated for 1 hour at 37°C. Wash the wells three times with tap  
20 water containing 0.05% Tween 80. Add per well 100 µl a 1:2000 fold dilution of a peroxidase-labeled rabbit anti-mouse immunoglobulin G conjugate in saturation buffer. Incubate for 1 hour at 37°C. Wash again three times with tap water containing 0.05% Tween 80. Add 100 µl of a  
25 standard solution of tetramethylbenzidine, sodiumacetate and hydrogenperoxidase to the wells and incubate for 10 minutes at room temperature. The reactions are blocked with 10% H2SO4. The various wells are examined at 450 nm, as standard.

30

Baculovirus and insect cells

The recombinant baculovirus AcRP23-lacZ (Bishop, 1992) was obtained from Dr. R. Possee, NERC Institute of Virology, Oxford, England, and the genomic DNA was  
35 purified as described by Summers and Smith (1987). Spodoptera frugiperda (Sf9) cell were obtained from the American Tissue Culture Collection (no. CRL 1711).

Baculovirus stocks were grown in confluent monolayers and suspension cultures in TC-100 medium (GIBCO/BRL) containing 10% fetal calf serum, as described by Summers and Smith (1987).

5

#### Cloning of CAV DNA.

All CAV DNA sequences are originally derived from the plasmid DNA pIc-20H/CAV-EcoRI (Noteborn and De Boer, 1990). All cloning steps with plasmid DNA were in principle carried out according to the methods described by Maniatis et al. (1982).

DNA transformations were carried out in the E.coli strain HB101. All plasmid were multiplied in large culture under agitation, purified on CsCl gradients, and then by filtration over Sephacryl-S500 columns or by filtrations on QIAGEN-chromatography columns.

#### Construction and selection of recombinant-VP1/VP2 baculovirus.

Recombinant transfer vector pAcVP1/VP2 DNA was transfected with linearized recombinant baculovirus AcRP23-lacZ DNA, in Sf9 cells. After homologous recombination baculoviruses were obtained, which had incorporated in the polyhedrin unit instead of the lacZ the two CAV proteins VP1 and VP2 under regulation of the promoter of the p10 or polyhedrin gene, respectively. In first instance, the recombinant CAV viruses were characterized for the absence of beta-galactosidase activity in plaques of baculovirus-infected insect cells. Further the integration of CAV DNA sequences in the baculovirus genome was determined by means of a CAV-specific DNA probe in a hybridization experiment.

#### Immunoprecipitation assay

Two days after infection, the cells were incubated with Promix label (ICN, USA) and four hours later, the cells were lysed in E1A buffer (50 mM Tris (pH7.5), 0.1%

Triton-X-100, 250 mM NaCl, 50 mM NaF, and 5 mM EDTA) and incubated with monoclonal antibody 111.1 directed against VP2 for 2 hours at 4°C, washed with E1A buffer and separated on a PAA-SDS gel.

5

#### Transfection of MDV DNA in CEFs.

For the construction of recombinant-VP1/VP2 MDV, co-transfections with purified recombinant-VP1/VP2 MDV-transfervector and DNA, isolated from chicken embryo  
10 fibro blasts (CEF) infected with MDV Rispens isolate (Van Vloten et al. 1972) were carried out according the method described by Graham and Van der Eb (1973).

#### Transfection of chicken T cells with mutant-CAV genomes.

15 The various mutated-CAV clones were digested with EcoRI and the EcoRI fragments containing the CAV (mutated) sequences was recircularized and transfected in MDCC-MSB1 cells, by using the DEAE-dextran method as has been described by Noteborn et al. (1991). As control, the whole  
20 procedure was in parallel carried out for the pCAV-EcoRI clone.

### RESULTS AND DISCUSSION

#### 25 The production of neutralizing monoclonal antibodies against CAV.

Noteborn and Koch (1994) have described two types of CAV-specific monoclonal antibodies. One type is directed against VP2, while the other is directed against VP3. None  
30 of these monoclonal antibodies reveal a CAV-specific neutralizing activity. Even, more important was that none of these monoclonal antibodies was directed against VP1. We assumed that a neutralizing monoclonal antibody might be directed against VP1, for the capsids contain mainly  
35 VP1 (Todd et al., 1990). Below, we describe the production of neutralizing antibodies against CAV.

For the production of neutralizing monoclonal antibodies against CAV, mice were injected with purified CAV particles.

As a first screening for (neutralizing) monoclonal antibodies against CAV, microtitre wells were coated with recombinant-VP1/VP2-baculovirus infected insect cells, which co-synthesized both VP1 and VP2 (see below). CAV-specific antisera with a high neutralizing titer reacted at a dilution of 1:1000 specifically with the recombinant VP1 and/or VP2 products (see below). Several different hybridoma cell lines producing monoclonal antibodies, which specifically reacted with recombinant VP1/VP2 products, were obtained.

A CAV-specific serum neutralization test, showed that three of these monoclonal antibodies obtained, had a neutralizing activity against CAV. These three CAV-specific neutralizing monoclonal antibodies were called 132.1; 132.2 and 132.3. (deposited under no's xxxx, yyyy, zzzz at the Institut Pasteur, France.

20

Microscopy examinations of the neutralizing monoclonal antibodies directed against CAV.

Immunofluorescence showed that the three neutralizing monoclonal antibodies 132.1, 132.2 and 132.3 recognize specific structures in CAV-infected MDCC-MSB1 cells. None of these monoclonal antibodies reacted with uninfected MDCC-MSB1 cells.

Electron-microscopic analysis was carried out with purified CAV particles incubated with neutralising antibodies against CAV (132.1) or with monoclonal antibodies 111.1 (against VP2) or 111.3 (against VP3). Todd et al. (1990) have reported that purified CAV capsids contain only a 50-kDa protein, which is most likely VP1. The various monoclonal antibodies were detected by immunogold labeling. Only the neutralizing monoclonal antibodies 132.1 were found to bind to CAV particles. Binding of the monoclonal antibody 132.1 to a CAV particle



resulted in its lysis. Furthermore, CAV capsids, which were lysed due to incubation with the neutralising monoclonal antibody 132.1, showed no binding with monoclonal antibodies directed against VP2 or VP3.

5        These results reveal the mechanism by which the neutralizing monoclonal antibodies act: They cause the lysis of the virus capsids, by doing so causing non-infectious particles. Furthermore, these data show that purified CAV particles contain (almost) only VP1.

10

Neutralizing monoclonal antibodies are directed against an conformational epitope on VP1.

      Pepscan analysis (Geysen et al., 1984) revealed that none of the 3 neutralizing monoclonal antibodies reacted  
15        significantly with one of the 12-mers derived from VP1 or VP2, or VP3. For the sake of brevity only the data obtained with monoclonal antibody 132.1 are shown for VP1 in Figure 1, and for VP2 in Figure 2. These results indicate that the neutralizing monoclonal antibodies are  
20        directed against a conformational epitope.

      These data were confirmed by the following experiments. Purified CAV particles, dotted on a nylon filter under native conditions, still could react with the neutralizing monoclonal antibody 132.1. However, after  
25        boiling in the presence of SDS, the CAV capsid proteins did not bind to monoclonal antibody 132.1.

      Immunoprecipitations experiments, carried under native conditions, as described by Noteborn et al. (1994b), with partially purified CAV particles and  
30        monoclonal antibody 132.1, 132.2 or 132.3 showed that a protein of about 50 kDa was precipitated by these monoclonal antibodies.

      It can be concluded that the presented results indicate that the neutralizing monoclonal antibodies are  
35        directed against an conformational epitope of VP1.

Enzyme-linked immunosorbens assay (ELISA) based on a neutralizing antibody against CAV.

We have developed a complex-trapping-blocking (CTB)-ELISA. One can use enriched CAV particles derived  
5 from CAV-infected MDCC-MSB-1 cells or recombinant VP1/VP2 proteins synthesized by means of the above described baculovirus system.

Serum from CAV-infected chickens contains antibodies which will block all epitopes on the CAV capsids or  
10 recombinant VP1/VP2. This means that the CAV capsid or recombinant VP1/VP2 will not bind to the coated monoclonal antibody 132.1. Negative serum, however, will allow binding of CAV capsids or recombinant VP1/VP2 to the coated 132.1. A signal smaller than 0.5 of the signal  
15 detected with a negative control serum will be examined as positive.

The detection level of our CTB-ELISA are titers of 24 to 25 as determined in a serum neutralization test, which is very sensitive. More than 400 sera were analyzed.  
20 Comparison to the serum neutralization test revealed that 96.5% of the positive sera within the serum neutralization test were positive within the CTB-ELISA, and 98.3% of the negative sera within the serum neutralization test were negative within the CTB-ELISA.

25

Construction of a recombinant-VP1/VP2 transfer vector.

The coding sequences for the CAV proteins VP1 and VP2 were cloned into the baculovirus transfer vector pAcUW51 (cat. no: 21205P), which was commercially obtained from  
30 PharMingen, San Diego, USA. This vector is shown in Figure 3 and contains the polyhedrin flanking region, within their midst the baculovirus polyhedrin promoter and the p10 promoter and for both transcription units, the required 3'-non-coding transcriptional sequences including the  
35 polyadenylation signals. The transfer vector contains prokaryotic sequences for multiplication in bacteria.

The plasmid pET-16b-VP2 (Noteborn et al., dates not published) contains CAV DNA sequences of positions 380 to 1512. This CAV DNA fragment contains the coding region for VP2 flanked by 484 bp 3'-non-coding CAV DNA sequences. 106  
5 bp downstream of the start codon for VP2 the start codon for VP3 is found in another reading frame. The plasmid pET-16b-VP2 was treated with the restriction enzymes NdeI and NheI, and the sticky ends were filled by means of Klenow polymerase. A 0.8 kb CAV DNA fragment was isolated.  
10 The plasmid pAcUW51 was linearized with BamHI, the sticky ends filled by means of Klenow polymerase and finally treated with alkaline phosphatase (CIP). The 0.8 kb CAV DNA fragment was ligated at the linearized pAcUW51 DNA. The orientation of VP2 in pAcUW51 was determined by  
15 restriction enzyme analysis. This construct was called pUW-VP2.

The plasmid pET-16b-VP1 (Noteborn et al., data not published) contains CAV DNA sequences of positions 853 to 2319. The CAV DNA insertion contains the complete coding  
20 region for the protein VP1 flanked by 117 bp 3'-non-coding CAV DNA sequences. The plasmid pET-16b-VP1 was treated with the restriction enzymes NdeI and EcoRI, and the sticky ends were filled by means of Klenow polymerase. A 1.45 kb CAV DNA fragment was isolated. The plasmid pUW-VP2  
25 was linearized by EcoRI, the sticky ends filled by means of Klenow polymerase and finally treated by CIP. The 1.45 kb CAV DNA fragment was ligated at the linearized pUW-VP2. The orientation of VP1 opposite of the p10 promoter unit was determined by restriction-enzyme analysis, and the  
30 final construct pAcVP1/VP2 is shown in Figure 3.

#### Construction of recombinant-VP1/VP2 baculovirus.

The open reading frames encoding VP1 and VP2 were separately cloned into a single baculovirus  
35 transfectant, viz pAcUW51. The CAV sequences encoding VP1 are under the regulation of the baculovirus p10 promoter and VP2 under the regulation of the baculovirus

poyhedrin promoter. Transfection of Sf9 insect cells occurred with 'naked' baculovirus DNA and transfervector DNA. After homologous recombination baculovirues were obtained which had incorporated the VP1/VP2 expression  
5 unit, integrated in the polyhedrin region of the recombinant baculovirus, instead of the lacZ unit. The recombinant baculoviruses were characterized for the absence of beta-galactosidase activity and integration of CAV DNA sequences.

10

Expression of the CAV proteins VP1 and VP2 in Sf9 cells.

The simultaneous expression of the CAV proteins VP1 and VP2 in Sf9 cells infected with recombinant-VP1/VP2 baculovirus was analyzed by Coomassie-brilliant blue  
15 staining and protein labeling with Promix (ICN, USA) or 3H-leucine (Amersham, UK) and PAA-SDS gel electrophoresis.

In the PCT/NL94/00168 it was shown that lysates of insect cells infected with recombi nant-VP1 baculovirus  
20 contained a CAV-specific protein of 52 kD and expression of VP2 by recombinant-VP2 baculovirus in infected insect cells produced a major specific product of 30 kDa. Infection of insect cells with recombinant-VP1/VP2 baculovirus resulted in the synthesis of both the  
25 CAV-specific proteins of 52 kD and 30 kD. Both CAV-specific products could be detected as radioactively labeled protein band or Coomassie-brilliant blue stained protein band. The latter result indicates that both products are produced in relatively high levels in recombi  
30 nant-VP1/VP2-baculovirus-infected insect cells. Sf9 cells infected with a recombinant-lacZ baculovirus did not contain these CAV-specific proteins.

Thus we have obtained evidence that inoculation in  
hens of crude lysates of recombinant-VP1/VP2-infected Sf9  
35 cells are able to induce neutralizing antibodies directed against CAV.

The role of VP2 for the formation of a conformational neutralizing epitope of VP1.

As reported in PCT/NL94/00168 and by Koch et al. (1995), simultaneous synthesis and not simply mixing of recombinant CAV proteins VP1 and VP2 is required to obtain a neutralizing and protective immune response. These data suggest that VP2 is a non-structural protein that at some stage of infection is required for virus assembly and/or the correct conformation of VP1, which result(s) in the formation of the neutralizing epitope(s). One explanation of the requirement of VP2 might be that it acts as a scaffold protein that is necessary during the assembly of the virion but absent in the final product (Leibowitz and Horwitz, 1975). Examples of scaffold proteins are the IVa2 and 39kDa proteins of adenovirus (D'Halluin et al., 1978; Persson et al., 1979). These proteins act as scaffolds for the formation of the so-called light capsid, but are removed in the next step. VP2 might function in a similar way during the formation of CAV virions. However, at this stage, we cannot entirely exclude that (very) small amounts of VP2 that remained undetected in electroblots of purified CAV preparations (Todd et al., 1990) or in electron microscopic photographs of lysed CAV particles incubated with immunogold-labeled VP2-specific monoclonal antibodies, as described above, associate with VP1 and form conformational neutralizing epitopes. Recently, weak evidence for the presence of VP2 in gradient-purified CAV was reported (Buchholz, 1994).

In the following experiments, evidence is provided that the neutralizing epitope of VP1 is only (optimal) present, when VP2 is simultaneously synthesized. Insect cells were infected with recombinant-CAV baculoviruses expressing VP1, VP2 (see PCT/NL94/00168) or both VP1 plus VP2. The infected Sf9 cells were harvested 3 or 4 days after infection and used for immunofluorescence tests with the CAV-specific neutralizing monoclonal antibody 132.1. The cells containing only the CAV-specific protein VP2 did

not react at all with the monoclonal antibody 132.1. Cells containing only VP1 revealed a very poor immunofluorescence signal after incubation with monoclonal antibody 132.1. However, insect cells infected with recombinant-VP1/VP2 baculovirus expressing both VP1 and VP2 bound very strongly to the neutralising monoclonal 132.1. PAA-SDS gel electrophoresis of in parallel radioactive-labeled lysates of insect cells expressing VP1, VP2 or VP1 plus VP2, revealed that VP1 is expressed at the same level when expressed only or simultaneously with VP2.

VP1 and VP2 interact temporarily with each other.

The neutralizing epitope of VP1 is only formed when VP2 is present, and therefore is a conformationally distinct epitope. This implies that VP1 and VP2 associate with each other during a short time period. By means of immunoprecipitations under very mild conditions, we have examined whether VP1 could associate with VP2. Sf9 insect cells were infected with recombinant baculoviruses, which synthesized VP1, VP2, or VP1 plus VP2.

The results clearly reveal that monoclonal antibody 111.1 precipitates VP2 when VP2 is synthesized alone or in the presence of VP1. In the case that besides VP2, VP1 was expressed also, VP1 co-precipitated to a small extent with VP2. The monoclonal antibody 111.1 did not detectably precipitate VP1, when VP1 was synthesized in the absence of VP2. These data indicate that VP1 and VP2 are (to a relatively small amount) associated to each other. During this association event, VP1 might obtain its conformation resulting in the neutralizing epitope.

Basis for the development of vaccines against CAV infections.

The above presented results show that for the induction of neutralizing antibodies against CAV, VP1 is required which has a specific conformation. In a



baculovirus expression system, this correct VP1 conformation is only possible, when VP1 plus VP2 or VP1 plus VP2 plus VP3 are simultaneously synthesized.

Denaturation of VP1 with e.g. sodium-dodecyl-sulphate  
5 result also in the destruction of its neutralizing epitope, indicating that the VP1 neutralizing epitope is a conformational one.

The recombinant CAV products, VP1 plus VP2 or VP1 plus VP2 plus VP3, which will be used for vaccination of  
10 laying-hens, can be synthesized by means of the baculovirus system. The CAV proteins can also be synthesized by means of other expression systems, such as yeast cells, via (retro)-viral infection or gene amplification (CHO-dhfr system) in mammalian cell systems.

15 In principle, the expression of fragments of VP1 (in combination with VP2 or VP2 and VP3) may be sufficient for the induction of a protective immune response. The fact that 12-mers of VP1 can not react with neutralizing antibodies against CAV indicates that larger VP1 fragments  
20 are needed for getting the correct VP1 conformation to form the neutralizing epitope. However, one should take into account that minor amino-acid mutations or a few amino-acid deletions might not influence the formation of the neutralizing epitope of VP1.

25 The fact that 2 or 3 proteins encoded by the CAV open reading frames can induce a protective immune response is also applicable to the development of living virus vectors. The coding sequences for VP1 plus VP2 or VP1 plus VP2 plus VP3 are then cloned into living virus vectors.

30 It is also possible that one of the CAV proteins, VP1, VP2 or VP3, separately, but then within the context of a living virus vector, is also suitable for the induction of a protective immune response against CAV infections. The expression of fragments of one of the above-mentioned CAV proteins by living virus vectors may be  
35 sufficient for the induction of a protective immune response.



The fact that VP3 causes apoptosis in, i.e. chicken mononuclear cells (PCT/NL94/00168; Noteborn et al., 1994a) favors to construct living virus vectors not expressing VP3. The replication of i.e. Marek's disease virus might  
5 be negatively influenced by VP3-induced apoptosis.

Alternatively, one can produce attenuated CAV expressing VP1 with the required conformation needed for eliciting a neutralizing and protective immune response against CAV infections.

10

Construction of a recombinant-MDV-VP1/VP2 transfer vector.

The coding sequences for the CAV proteins VP1 and VP2 were cloned into the Marek's disease virus (MDV) transfervector pMD-US10-SV (Koch, unpublished data). The  
15 transfervector contains the SV40 early promoter, which are flanked by sequences of the MDV US10 region, and prokaryotic sequences for multiplication in bacteria. A schematic representation of the transfer vector is given in Figure 4.

20

The CAV coding sequences from position nt 368 to nt 2319 were inserted in the pMD-US10-SV transfervector under the regulation of the SV40 early promoter. This recombinant transfervector was checked by restriction enzyme digestion and is called pMD-US10-SV-VP1/VP2.

25

By introducing a point-mutation at position 549 (T was changed into an A) an extra stopcodon has been introduced in the gene encoding VP3 (Noteborn, unpublished data). Therefore, the inserted CAV sequences will only express VP1 and VP2. Indirect-immunofluorescence analysis  
30 of chicken embryo fibroblasts (CEF) transfected with the vector pMD-US10-SV-VP1/VP2 proved that VP1 and VP2 were indeed expressed, whereas VP3 was not.

Construction of recombinant-VP1/VP2 MDV.

35

Using the Ca-phosphate transfection method, recombinant transfer vector pMD-US10-SV-VP1/VP2 DNA was transfected with MDV (Rispen's isolate) DNA, in CEF cells.

After homologous recombination, recombinant MDVs were obtained, which had incorporated the CAV coding sequences in the the US10 region (Nakamura et al., 1992). The recombinant-VP1/VP2 MDVs were characterized for the presence of the expression of VP2. By in-parallel plaque purification and immuno-peroxidase analysis using a monoclonal antibody directed against VP2, several 100%-purified recombinant-VP1/VP2 MDV independent strains were obtained.

10

#### Characterization of recombinant-VP1/VP2 MDV.

The correct integration of the CAV-DNA sequences in the MDV genome was determined by means of PCR and restriction-enzyme analysis. By subsequent passaging of the various purified recombinant-VP1/VP2 MDVs, it was shown that they remained stable. No recombinants were obtained with (partial) loss of the VP1/VP2 expression unit.

The simultaneous expression of the CAV proteins VP1 and VP2 in CEF cells, which were infected with the recombinant-VP1/VP2 MDV, was proven by indirect immunofluorescence. The monoclonal antibodies CVI-CAV-111.1 (111.1), directed against VP2 and CVI-CAV-132.1 (132.1), directed against VP1 were used.

The monoclonal antibody 132.1 is directed against a neutralizing epitope of VP1. The fact that the neutralizing antibody 132.1 can recognize recombinant-MDV expressed VP1, co-synthesized with VP2, implies that the required neutralizing epitope is present on MDV-expressed VP1. Therefore, vaccination of chickens with recombinant-VP1/VP2 MDV will result in the induction of a neutralizing/protective antibody response. Besides the synthesis of the CAV VP1 and VP2 proteins, the recombinant-VP1/VP2 MDV will synthesize the MDV proteins required for the induction of a protective immune response against MDV infections.

Production of attenuated CAV based on the cloned CAV genome pCAV/EcoRI.

The CAV-EcoRI clone is used for the production of viable infectious CAV particles. To that end, the complete  
5 CAV insert, the 2,319-bp EcoRI fragment has to be isolated from the bacterial sequences and recircularized by DNA polymerase treatment. Subsequently, e.g. MDCC-MSB1 cells are transfected with the recircularized EcoRI fragments and after several days wild-type CAV will be produced.

10 One can introduce mutational changes in the CAV sequences of the CAV-EcoRI clone and isolate, recircularize and transfect in MDCC-MSB1 cells. Instead of wild-type CAV, in principle one can produce mutant CAV and if the mutant is less pathogenic, attenuated CAV. The  
15 complete strategy for the production of attenuated CAV is shown in Figure 5.

Construction of CAV genomes containing mutated promoter/enhancer regions.

20 A remarkable feature of the CAV promoter/enhancer region is a sequence of 4 or 5 near-perfect 21-bp repeats, interrupted by an insert of 12-bp (Noteborn et al., 1991). This region is involved in the regulation of the CAV transcription (Noteborn et al., 1994b).

25 For the production of attenuated CAV, we have introduced mutational changes in the 12-bp insert/direct-repeat region of the CAV-EcoRI clone (Noteborn et al., 1991). The mutated promoter/enhancer region of the wild-type (wt) and the various CAV mutants  
30 are shown in Fig. 6. The Nae-mutant contains no 12-bp/direct-repeat region sequences at all. Instead of these, a NaeI site (5'-GCCGGC-3') has been introduced, which is given as 'N' in figure 6.

All other mutants contain 4 original direct repeats  
35 instead of 5, as is the case for the CAV-EcoRI clone. The various CAV mutants, called '6b'p, '12bp', '24bp' contain changed 12-bp inserts. The mutant 'wt in Nae' and of

course the original CAV-EcoRI clone, called 'wt', contain the original 12-bp insert sequences 5'-AAGAGGCGTTCC-3'.

The CAV mutants '6bp', '12bp', '18bp', '24bp' and 'wt in Nae' contain all additional sequences flanking the  
5 12-bp insert/direct-repeat region. At the 5'-site of this region, the linker 5'-GCCCATGT-3', and at the 3'-site the linker 5'-GATCCGCC-3' has been introduced.

10 Mutated CAV genomes results in the production of attenuated infectious CAV.

To determine whether the mutated CAV genomes could produce live CAV particles, firstly the synthesis of VP3 was examined. At several timepoints after transfection, a part of the various transfected MDCC-MSB1-cell cultures  
15 were acetone-fixed and analysed by indirect immunofluorescence using a monoclonal antibody directed against VP3. In parallel, 1 ml of each culture was added to 9 ml of fresh RPMI-medium, which was supplemented with 10% fetal bovine serum.

20 Approximately 15% and 90% of the cultured MDCC-MSB1 cells, transfected with 'wt' recircularized CAV genome DNA, contained VP3 after 6 days and 11 days (the cells were one time passaged after transfection), respectively.

The CAV mutant genome 'Nae', lacking the complete  
25 12-bp insert/direct-repeat region, was shown not to produce infectious virus particles. Six days after transfection, at most 2% of the cells produced VP3, which is due to transient expression of the 'Nae' DNA. A similar percentage is obtained with expression plasmid pRSV-VP3  
30 (Noteborn et al., 1994b) encoding VP3 only, and which are known not to replicate in eukaryotic cells. After 3 weeks and several passages, no VP3 was present anymore in the Nae-DNA transfected MDCC-MSB1 cell cultures.

At various time-intervals after transfection, the  
35 percentage of VP3-positive cells of the MDCC-MSB1 cultures, transfected with the mutant-DNA genomes '6bp', '12bp', '18bp', '24bp' and 'wt in Nae' was significantly lower

compared with cultures, transfected with 'wt'-DNA genome. These data imply that the various CAV mutants replicate less fast than the wild-type CAV. Especially, the mutants '6bp' and '18bp' were reduced in their replication rate.

5 Most likely, the mutational changes of the 12-bp insert results in the lower virus spread of the mutated CAV. The results of these experiments are shown in Figure 7.

Subsequently, we have infected fresh MDCC-MSB1 cultures with supernatants of cells transfected with all  
10 examined mutated CAV-DNA genomes. All supernatants, except the one derived from 'Nae'-DNA-transfected cells, were proven to contain infectious virus particles. For these cultures, infected with the 'mutated-virus' containing supernatants, it could be shown by indirect  
15 immunofluorescence, that cells were present containing VP3.

#### CAV mutants have a reduced cytopathogenic effect.

In parallel with the above described detection of VP3  
20 synthesis, the transfected cells were analysed whether they had become apoptotic. CAV is known to induce apoptosis, 2-3 days after infection. To that end, the cells were stained with propidium iodide, which stains 'normal' DNA strongly, but apoptotic DNA weakly and/or  
25 irregularly. Already, 11 days after transfection almost all MDCC-MSB1 cells transfected with 'wt' DNA were shown to be apoptotic, whereas the majority of the cells, transfected with the various mutated CAV-DNA genome were not. The capacity for inducing apoptosis was shown to be  
30 mostly reduced for the CAV mutants '6bp' and '18bp'.

#### DNA analysis of attenuated CAV.

By polymerase-chain reaction and sequence analysis of the 12-bp insert/direct-repeat region and flanking  
35 sequences, it was shown that the original mutations were not changed for the various CAV mutants '6bp', '12bp', '18bp', '24bp' and 'wt in Nae'. These results indicate

that the various CAV mutants are stable, at least after culturing in MDCC-MSB1 for about 1 month and that indeed these CAV mutants are viable and causing a reduced cytopathogenic effect in cultured chicken T cells.

5       Southern blot analysis of DNA isolated from MDCC-MSB1 cultures, infected with the various CAV mutants, revealed that all viable CAV mutants produced all CAV DNAs, as was described for wild-type CAV. The double-stranded replication intermediate and single-stranded DNA were  
10 detected for both wild-type and mutated CAV.

Attenuated CAV synthesizes the CAV-specific neutralizing epitope.

By indirect-immunofluorescence, using the  
15 neutralizing monoclonal antibody CVI-CAV 132.1 and a monoclonal antibody directed against VP2, viz CVI-CAV 111.2 and VP3, viz CVI-CAV-85.1, it was shown that all mutated CAV genomes were able to synthesize the CAV proteins VP1, VP2 and VP3. Even more important is that the  
20 neutralizing monoclonal antibody 132.1 reacted with cells, infected with the various CAV mutants. This finding indicates that the mutated CAVs will produce the required neutralizing CAV-specific epitope, which has to be recognized by the immune system of vaccinated chickens as  
25 CAV for eliciting a protective immune response.

It can be concluded that based on the CAV clone pCAV-EcoRI, mutated CAV genomes can be made, resulting in the production of viable mutated CAV particles. These CAV mutants replicate in cultured chicken T cells less fast  
30 than wild-type CAV. The cytopathogenic effect caused by these CAV mutants is also reduced compared to wild-type CAV. The CAV mutants are all able to produce all CAV proteins resulting in the required neutralizing epitope on VP1.

35       Therefore, these CAV mutants are attenuated versions of CAV in the field.

Description of the Figures

Figure 1 shows the pepscan analysis of the neutralizing monoclonal antibodies of type 132.1 with peptides (12-mers) derived from VP1.

5        Figure 2 shows the pepscan analysis of the neutralizing monoclonal antibodies of type 132.1 with peptides (12-mers) derived from VP2.

Figure 3 shows the diagrammatic representation of the recombinant transfer pUW-VP1/VP2.

10       Figure 4 shows the diagrammatic representation of the recombinant transfer vector pMD-US10-SV-VP1/VP2..

Figure 5 shows the strategy for the production of attenuated CAV-mutants, based on the plasmid pCAV/EcoR1 containing 2319 bp of wild-type CAV.

15       Figure 6 shows the schematic structure of the CAV promoter/enhancer region of the various mutated and wild-type CAV strains.

Figure 7 shows the VP3-expression rates obtained for the various mutant-CAVs and wild-type CAV after  
20       transfection of mutant or wild-type circularized DNA. The VP3-expression rate are given as percentage of VP3-positive MDCC-MSB1 cells.

Figure 8 gives the sequence of CAV.



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CLAIMS

1. A neutralizing antibody or an antibody fragment or derivative thereof reacting with a conformational epitope of viral protein 1 (VP1) of chicken anaemia virus (CAV) recognized by a monoclonal antibody designated as 132-1,  
5 132-2 or 132-3, as produced by the hybridoma's deposited under no.'s xxxxxx, yyyyyy, zzzzzz at the Institut Pasteur, Paris, France.
2. A conformational neutralizing epitope of viral protein 1 of chicken anaemia virus recognized by an  
10 antibody according to claim 1.
3. A method for producing a viral protein 1 comprising a conformational epitope according to claim 2, comprising the expression of said viral protein 1 in one cell together with viral protein 2 of said CAV whereby the  
15 genetic information encoding VP1 and the genetic information encoding VP2 are separately present on one recombinant vector.
4. A vector for use in a method according to claim 3 comprising as two separate coding sequences the genetic  
20 information encoding VP1 and the genetic information encoding VP2.
5. A vector according to claim 4 which is based on Marek's disease virus vector.
6. A vector according to claim 4 which is based on a  
25 baculo virus vector.
7. A method for providing a viral protein 1 comprising a neutralizing conformational epitope according to claim 1, comprising expressing at least a functional part of VP1 and VP2 from genes encoding them, which genes are under  
30 control of a regulatory sequence derived from the CAV sequence upstream of the transcription intitiation site which regulatory sequence is modified to reduce its efficiency.



8. A method according to claim 3 or 7 whereby VP1 is provided in the form of virus particles.
9. A method according to claim 7 whereby the modification is in the promoter/enhancer region.
- 5 10. A method according to claim 9 whereby the modification is in the 12 base pair insert in the promoter enhancer region.
11. Recombinant virus particles obtainable by a method according to claim 7 -10.
- 10 12. A nucleic acid for use in a method according to claim 7 -10 comprising a gene encoding at least a functional part of VP1 and a gene encoding a functional part of VP2, at least one of the genes being under control of a regulatory sequence which is modified to reduce its
- 15 efficiency.
13. A diagnostic test kit for detecting or determining the presence of CAV or antibodies to CAV in a sample using an antibody according to claim 1 and/or an epitope according to claim 2.
- 20 14. A vaccine for the treatment or prophylaxis of CAV associated disease comprising an antibody according to claim 1 or an epitope according to claim 2 together with a suitable adjuvans and/or a suitable vehicle for administration.
- 25 15. A vaccine for the treatment or prophylaxis of CAV associated disease comprising recombinant virus particles according to claim 11 together with a suitable adjuvans and/or a suitable vehicle for administration.

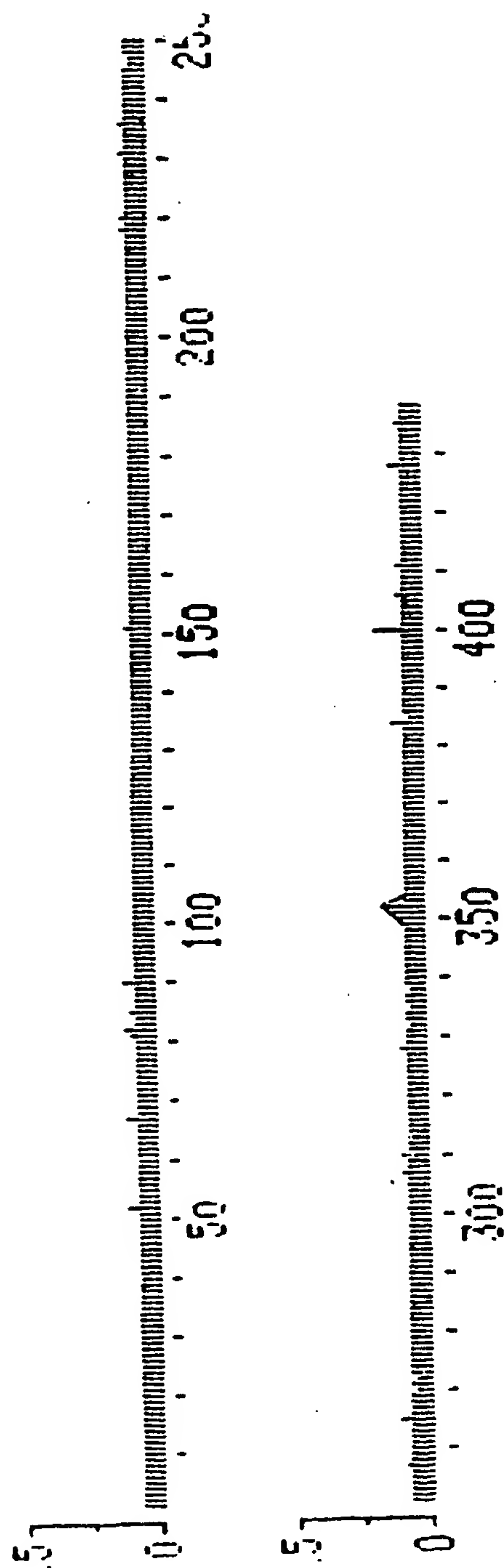


FIG. 1

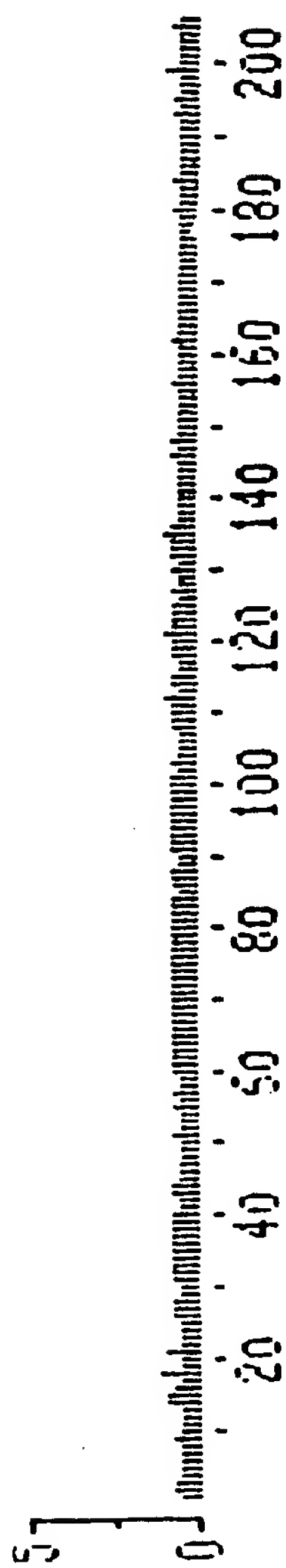


FIG. 2

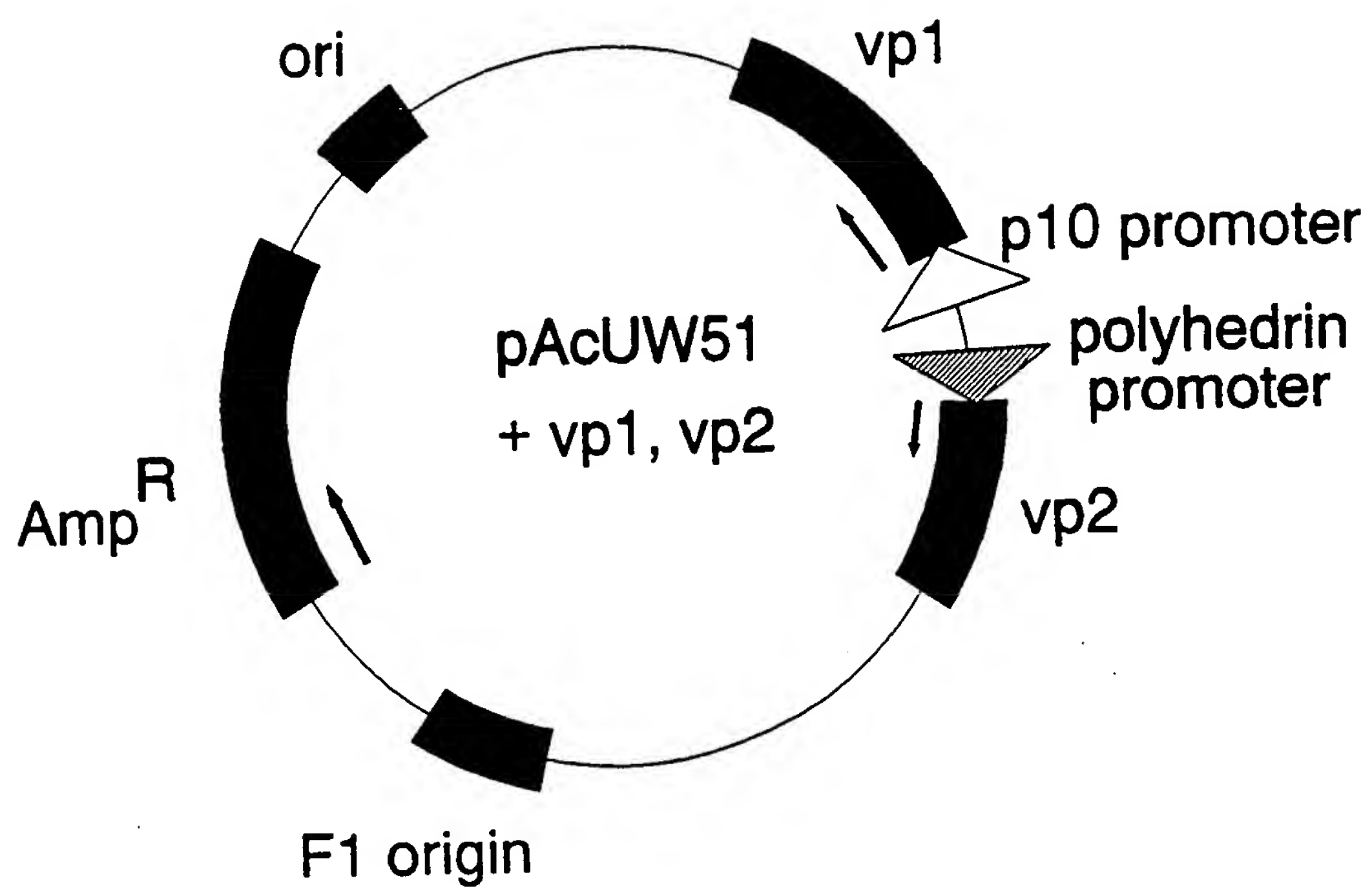
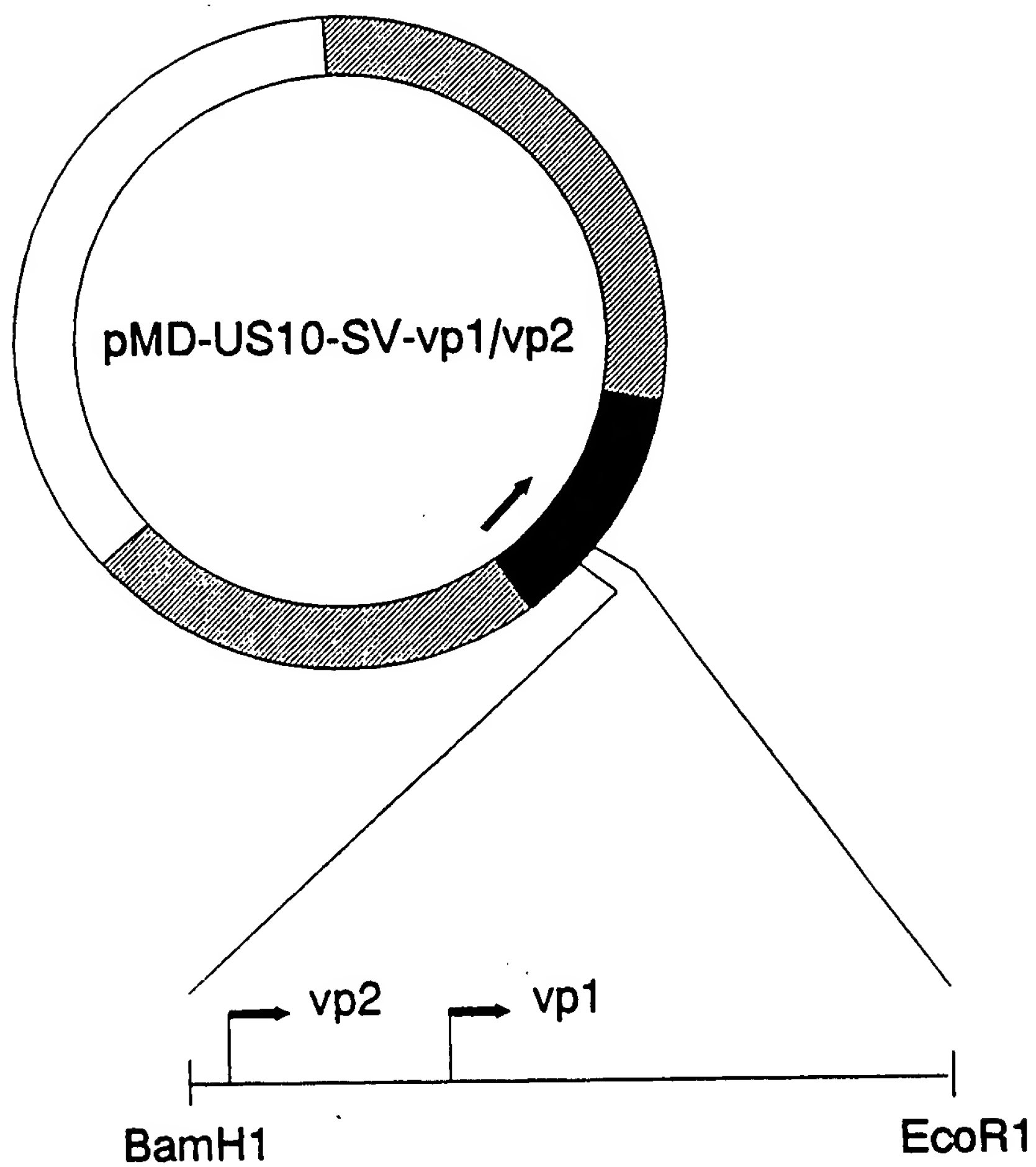


FIG. 3

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-  : US10-flanking
-  : SV40 seq.
-  : SV40 promoter
-  : ATG startpoint

FIG. 4

## Attenuated CAV

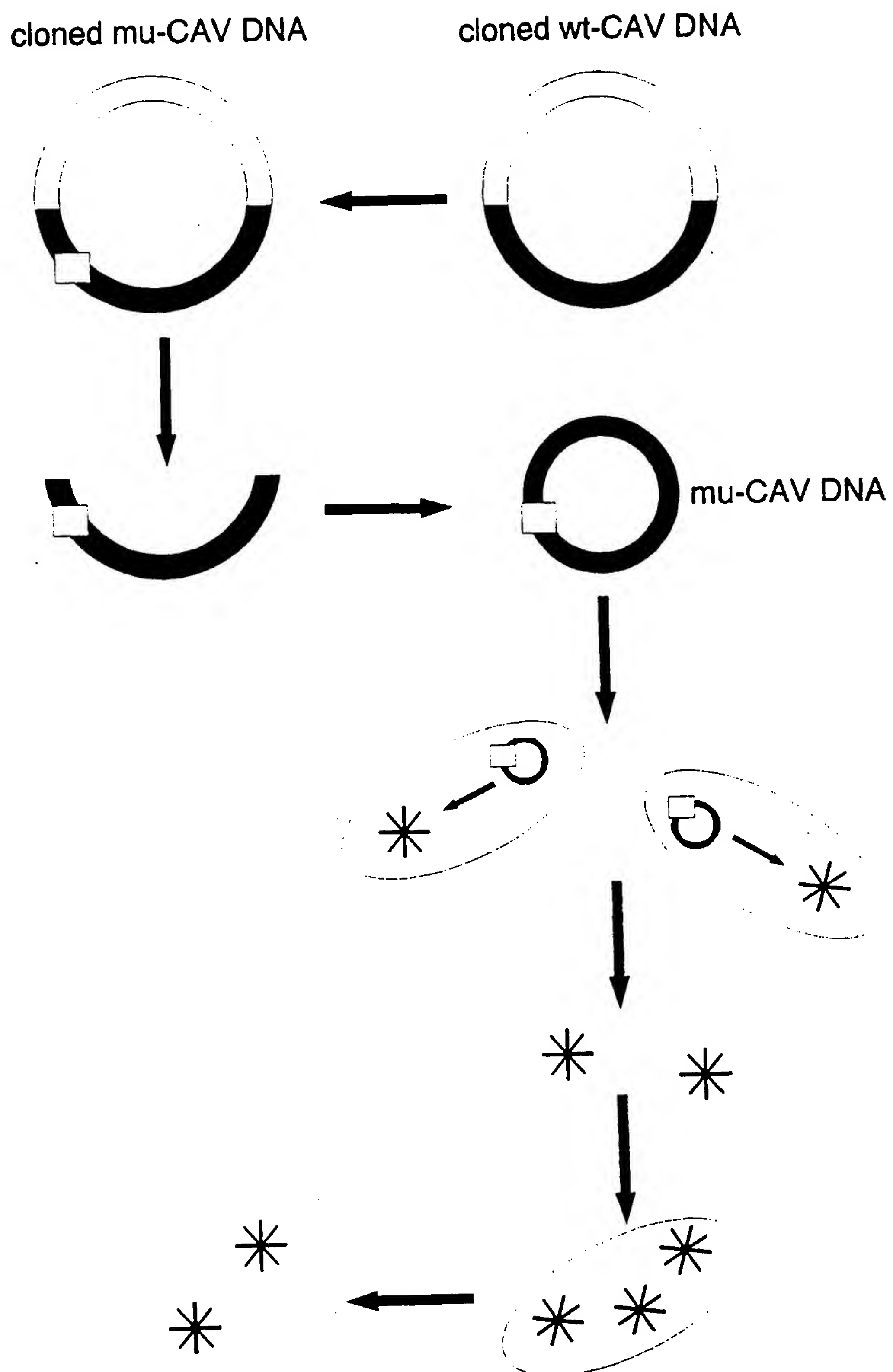


FIG. 5

## Promoter/enhancer region of wt en mu CAV strains

	$N_L$	1A 1B 2		3 4	$N_R$
Nae	$N_L$				$N_R$
6 bp	$N_L$	1B 2	GTCGAC	3 4	$N_R$
12 bp	$N_L$	1B 2	AGCGCAGTCGAC	3 4	$N_R$
18 bp	$N_L$	1B 2	GTCGATAGCGCGCTCGAC	3 4	$N_R$
24 bp	$N_L$	1B 2	GTCGACAAGAGGCGTTCCGTCGAC	3 4	$N_R$
wt in Nae	$N_L$	1B 2	AAGAGGCGTTCC	3 4	$N_R$
wt		1A 1B 2	AAGAGGCGTTCC	3 4	

FIG. 6



# CAV mutants

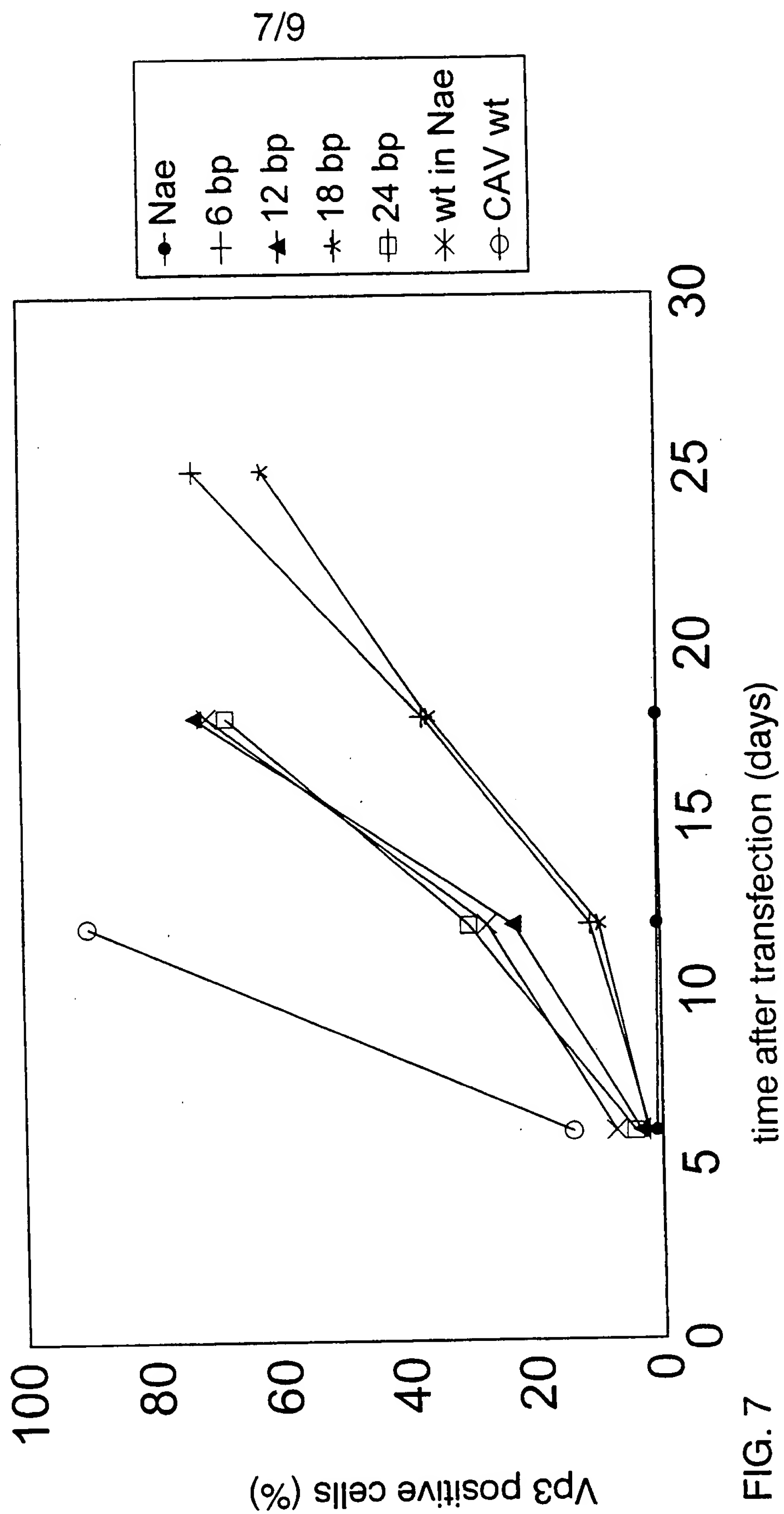


FIG. 7

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10 GAATTCCGAG TGGTTACTAT TCCATCACCA TTCTAGCCTG TACACAGAAA GTCAAGATGG ACGAATCGCT CGACTTCGCT CGCGATTTCGT CGAAGGCCGG 100

110 GGGCCGGAGG CCCCCCGGTC CCCCCCCTCC AACGAGTGA GCACGTACAG GGGCGTACGT CATCCGTACA GGGGGGTACG TCATCCGTAC AGGGGGGTAC 200

210 GTCACAAAGA CGCGTTCCCG TACAGGGGGG TACGTACCGC GTACAGGGGG GTACGTCACA CCCAATCAAA AGCTGCCACG TTGCGGAAAGT GACGTTTCGA 300

310 AAATGGCGG CGCAAGCCTC TCTATATATT GAGCGCACAT ACCGGTCGGC AGTAGGTATA CGCAAGGCGG TCCGGGTGA TGCACGGGAA CGGCGCACAA 400

410 CCGGCCGCTG GGGGCAGTGA ATCGGCGCTT AGCCGAGAGG GGCAACCTGG GCCCAGCGGA CCCGGGCAGG GGCAAGTAAT TTCAAAATGAA CGCTCTCCAA 500

510 GAAGATACTC CACCCGGACC ATCAACGGTG TTCAGGCCAC CAACAAGTTC ACGGCCGTTG GAAACCCCTC ACTGCACAGA GATCCGGATT GGTATCGCTG 600

610 GAATTACAAT CACTCTATCG CTGTGTGGCT GCGGGAATGC TCGCGCTCCC ACGCTAAGAT CTGCAACTGC GGACAATTCA GAAAGCACTG GTTTCAGAA 700

710 TGTGCCGGAC TTGAGGACCG ATCAACCCAA GCCTCCCTCG AAGAAGCGAT CCTGCGACCC CTCCGAGTAC AGGTPAAGCG AGCTAAAAGA AAGCTTGATT 800

810 ACCACTACTC CCAGCCGACC CCGAACCGCA AAAAGGCGTA TAAGACTGTA AGATGGCAAG ACGAGCTCGC AGACCGAGAG GCCGATTTTA CTCCCTTCAGA 900

910 AGAGGACGGT GGCACCCACCT CAAGCGACTT CGACGGAAGAT ATAAATTTCG ACATCGGAGG AGACAGCGGT ATCGTAGACG AGCTTTTAGG AAGCCCTTTC 1000

1010 ACAACCCCGG CCCCCGTACG TATAGTGTGA GGCTGCCGAA CCCCCAATCT ACTATCACTA TCCGCTTGCA AGGGTTCATC TTCTCAGCG AAGGACTCAT 1100

1110 TCTGCCTAAA AACAGCACAG CGGCGGGCTA TGCAGACCAC ATGTACGGGG CGAGAGTCGC CAAGATCTCT GTGAACCTGA AAGAGTTCCT GCTAGCCCTCA 1200

FIG. 8(1/2)

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1210	1220	1230	1240	1250	1260	1270	1280	1290	1300
ATGAACCTGA	CATACGTGAG	CAAAATCGGA	CGCCCCATCC	CCGGTGAGTT	GATGCCCCAC	GGGTCTAAAT	CACAACCCCG	GGACAATTGG	CCTAATTGCT
1310	1320	1330	1340	1350	1360	1370	1380	1390	1400
GGCTGCCGCT	AGATAATAAC	GTGCCCTCCG	CTACACCATC	GGCATGGTGG	AGATGGGCCT	TAATGATCAT	GCAGCCCCACG	GACTCTTGCC	GGTCTTTAA
1410	1420	1430	1440	1450	1460	1470	1480	1490	1500
TCACCCAAAG	CAGATGACCC	TGCAAGACAT	GGGTCCCATG	TTTGGGGCCT	GGCACCTGTT	CCGACACATT	GAAACCCCGCT	TTCAGCTCCT	TGCCACTAAG
1510	1520	1530	1540	1550	1560	1570	1580	1590	1600
AATGAGGGAT	CCTTCAGCCC	CGTGGCGAGT	CTTCTCTCCC	AGGAGAGTA	CCTCACGCGT	CGGGACGATG	TTAAGTACAG	CAGCGATCAC	CAGAACCCGGT
1610	1620	1630	1640	1650	1660	1670	1680	1690	1700
GGCAAAAAGG	CGGACAACCG	ATGACGGGGG	GCATTGCTTA	TGCGACCCGG	AAAATCAGAC	CCGACGACCA	ACACTACCCT	GCTATGCCCC	CAGACCCCCC
1710	1720	1730	1740	1750	1760	1770	1780	1790	1800
GATCATCACC	GCTACTACAG	CGCAAGGCAC	GCAAGTCCGC	TGCATGAATA	GCACGCAAGC	TTGGTGGTCA	TGGCACACAT	ATATGAGCTT	TGCAACACTC
1810	1820	1830	1840	1850	1860	1870	1880	1890	1900
ACAGCACTCG	GTGCACAATG	GTCTTTTCT	CCAGGGCAAC	GTTCAAGTTT	TAGACGGTCC	TTCAACCACC	ACAAGGCGAG	AGGACCCCGG	GACCCCAAGG
1910	1920	1930	1940	1950	1960	1970	1980	1990	2000
GCCAGAGATG	GCACACGCTG	GTGCCGCTCG	GCACGGACAC	CATCACCCGAC	AGCTACATGT	CAGCACCCCG	ATCAGAGCTG	GACACTAATT	TCTTTACGCT
2010	2020	2030	2040	2050	2060	2070	2080	2090	2100
TTACGTAGCG	CAAGGCACAA	ATAAGTCGCA	ACAGTACAAG	TTCGGGCACAG	CTACATACGC	GCTAAAGGAG	CCGGTAATGA	AGAGCGATGC	ATGGGCACTG
2110	2120	2130	2140	2150	2160	2170	2180	2190	2200
GTACGCGTCC	AGTCGGTCTG	GCAGCTGGGT	AACAGGCAGA	GGCCATACCC	ATGGCACGTC	AACTGGGCGA	ACAGCACCAT	CTACTGGGGG	ACGCAGCCCT
2210	2220	2230	2240	2250	2260	2270	2280	2290	2300
CAAAAGGGGG	GGGGGCTAAA	GCCCCCCCCC	CTTAAACCCC	CCCCCTGGGG	GGATTCCCCC	CCAGACCCCC	CCTTTATATA	GCACTCAATA	AAGGCAGAAA
2310									
ATAGATTAT	CCCACTAC								

FIG. 8(2/2)

## INTERNATIONAL SEARCH REPORT

Int. Application No  
PCT/NL 96/00230

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/34 C07K16/08 C07K14/01 C12N15/86 G01N33/569  
G01N33/577 A61K39/42 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 95 03414 A (AESCULAAP B.V.) 2 February 1995 cited in the application see examples see claims --- -/-	1-4,6, 13,14

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

16 September 1996

Date of mailing of the international search report

27.09.96

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## INTERNATIONAL SEARCH REPORT

Int. onal Application No

PC1/NL 96/00230

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

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PCT/NL 96/00230

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